Supporting Information

Prodigiosin is a chloride carrier that can function as an anion exchanger

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Materials. DPPC and EYPC lipids were purchased from Avanti Polar Lipids. Polycarbonate membranes and the extrusion apparatus used for making the liposomes was also from Avanti Polar Lipids.[1] Salts (> 99% purity) were purchased from Sigma-Aldrich and used as received. Fluorescent dyes HPTS and Lucigenin were purchased from Fluka and Molecular Probes, respectively. Prodigiosin **1** was generously given to us by Dr. Richard Manderville, which he obtained from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis of the NCI.

Buffer solutions were made from ultra-pure water (distilled and then passed through a Millipore filtering system). The pH adjustments, though rarely needed, were made using concentrated NaOH or phosphoric acid solution. Fluorescence experiments for the DPPC and pH dependence assays were completed on a Hitachi model F-4500 fluorescence spectrophotometer equipped with temperature control maintained by a Brinkman water-bath circulator (temperature maintenance was +/- 0.2 ^{o}C). The EYPC comparative assays were performed using a Fluoromax 3 (Jobin-Yvon/Horriba) spectrophotometer at ambient temperature.

Preparation of DPPC liposomes. DPPC lipid (50 mg) was dissolved in 5 mL of a chloroform/methanol mixture (5 % MeOH), and the resulting solution was then evaporated under reduced pressure at 45 ^{o}C to produce a thin film that was then dried *in vacuo* for 2 h. The lipid film was hydrated with 1mL of solution of 10 mM sodium phosphate (pH=6.4) containing 100 mM $NaNO_3$ and 1 mM lucigenin dye.[2] After 5 freeze/thaw cycles (thawing, and then warming to 45 ^{o}C) the liposomes were extruded

through a 100 nm polycarbonate membrane 21 times at temperature between 45-55 ^{o}C (fluid state lipid). The liposome solution was then passed through a Sephadex (G-25) column to remove any excess lucigenin dye. The isolated liposomes were diluted in 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a final concentration of 11 mM in DPPC lipid, assuming 100 % retention of lipid during the gel filtration process.

Chloride Transport Assay in DPPC Liposomes. In a typical experiment, 0.1 mL of the stock DPPC liposome solution was diluted into 2 mL of a solution of 10 mM sodium phosphate (pH 6.4, 100 mM $NaNO_3$) to give a solution that is 0.5 mM in lipid. To this solution was added 4 μL of a 0.01 mM solution of prodigiosin **1**, giving a 1:25,000 ligand to lipid ratio (or 0.004 mol %). Fluorescence was monitored at excitation 372 nm and emission at 504 nm for 500 s. At t=20 s, .02 mL of 4.0 M NaCl was added to the cuvette through an injection port to give a final concentration of 25 mM chloride outside of the liposomes. After 470 s, 0.04 mL of 10 % Triton-X detergent was added to destroy the liposomes and determine maximal fluorescence quenching of lucigenin by Cl^-. Experiments at two different temperatures (37 ^{o}C and 43 ^{o}C) were each repeated in triplicate and all traces reported are the average of the three trials.

Lucigenin fluorescence was converted to chloride concentration using the Stern-Volmer constant determined under the assay conditions.[2] To measure the Stern-Volmer constant, liposomes were prepared as above, except that the liposomes were lysed immediately with Triton-X. Then, 5 μL of 4.0 M NaCl was titrated in every 30 s via the injection port. The titration was completed twice. A plot of f_0/f vs. chloride

concentration was generated (**Figure S1**), the slope of which is taken to be the Stern-Volmer constant.

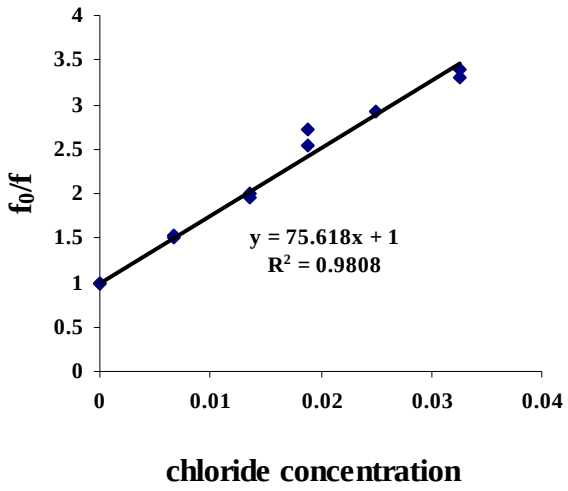

Figure S1. Determination of the Stern-Volmer constant for lucigenin quenching by Cl^-.

DPPC assay using a calixarene tetramide 2 as a positive control for prodigiosin 1. We compared the chloride transport of prodigiosin **1** with the activity of another chloride transporter (the *partial cone* calix[4]arene tetrabutylamide **2**) at 43 ^oC and 37 ^oC. Data for prodigiosin is reported in Figure 1 of the communication, while data for calixarene **2** is shown in **Figure S2**. We have previously reported that an analog of calixarene **2** (the *1,3 alternate* conformer) forms channels in lipid bilayers.[3] We find that **2** also forms channels, and a full report on the properties of **2** and analogs is forthcoming.

We find that 2 mol % of calixarene **2** transports chloride at 43 ^oC at approximately the same rate as 0.004 mol % prodigiosin **1**. At 37 ^oC, below the gel to liquid crystalline transition temperature for DPPC, calixarene **2** still shows significant transport activity, while prodigiosin **1** does not transport chloride at this temperature. The

complete depletion of Cl^- transport by prodigiosin **1** at 37 ^oC is evidence for a carrier mechanism. [4]

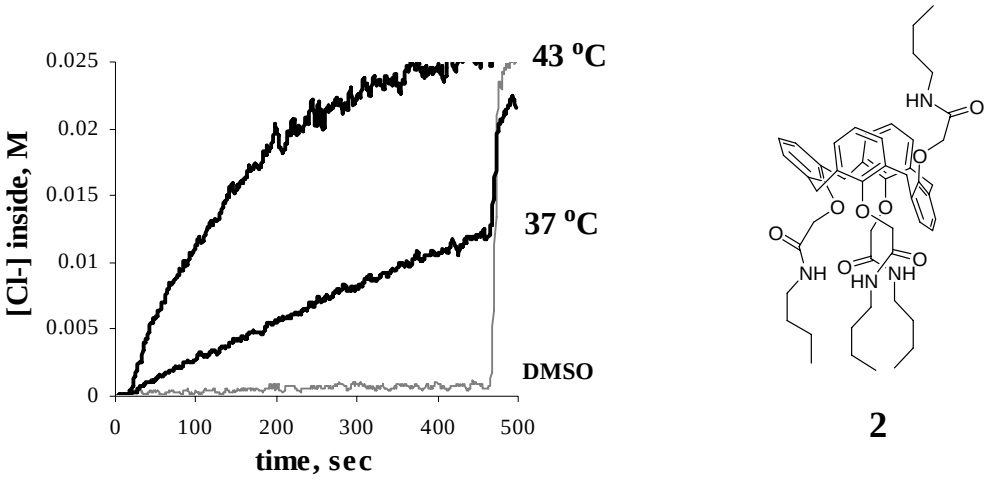

Figure S2. Chloride transport in DPPC liposomes via our own synthetic anion transporter, *partial cone* calix[4]arene tetrabutylamide **2**

Determination of chloride transport as a function of prodigiosin concentration in EYPC liposomes. A stock liposome solution was prepared as described above, except EYPC lipid was substituted for DPPC. Experiments were conducted as described above such that the final lipid concentration was 0.5 mM in 2 mL of 10 mM sodium phosphate, 100 mM $NaNO_3$ (pH 6.4) with concentrations of prodigiosin **1** ranging from 0.01 to 0.0005 μM. The lucigenin fluorescence was monitored for 300 s. The addition of chloride (final concentration 25 mM) was made at t=20 s, and the liposomes were lysed with Triton-X at t=250 s. Fluorescence was converted to chloride concentration using the Stern-Volmer constant as calculated above. Pseudo first-order rate constants were determined from the graph of $\ln([Cl^-]_{out}-[Cl^-]_{in})$ vs. time where $[Cl^-]_{out}$ is calculated from

the final point of the fluorescence data. The plot of k_{obs} vs. time is linear (**Figure S3**), consistent with prodigiosin being a transmembrane carrier.[3]

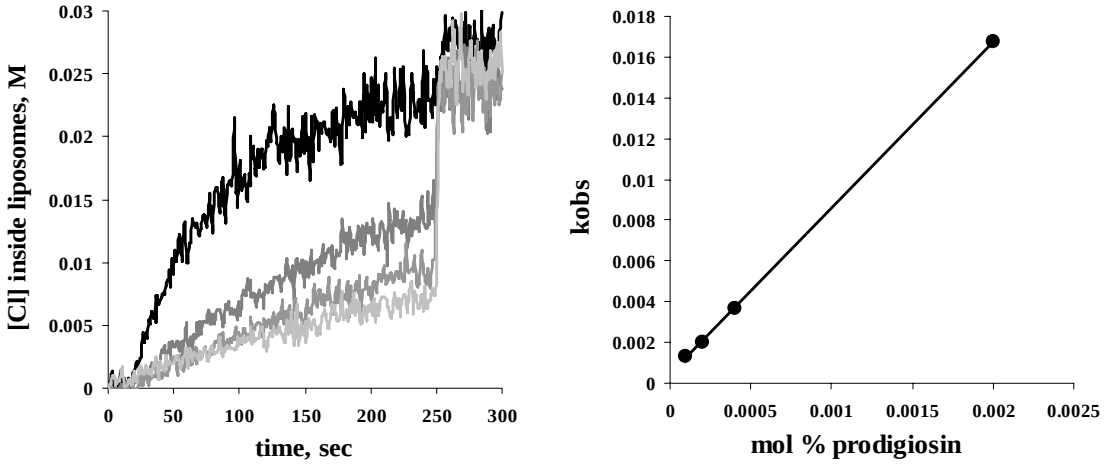

Figure S3. Chloride transport as a function of the concentration of prodigiosin **1** in EYPC liposomes, as determined by fluorescence of lucigenin dye.

Chloride transport by prodigiosin 1 at different pH. EYPC liposomes containing 1 mM lucigenin dye were prepared as described for the DPPC assay, except that they were prepared at room temperature. The lipid was hydrated with, and suspended in a solution of 10 mM sodium phosphate, 75 mM Na_2SO_4 at pH of either 5.4 or 7.4. A sodium chloride gradient (final concentration 25 mM) was applied as described for previous assays, and the lucigenin fluorescence was monitored as chloride entered the liposomes. The fluorescence data was converted to pseudo first-order rate constants as previously described. The results of the assays are shown below in **Figure S4**. There is a pH dependence on the rate of chloride transport by prodigiosin **1**. These observations are in

agreement with results previously reported for prodigiosin analogs, which also showed lower transport rates at more acidic pH.[5] The influence of pH on the Cl^- transport rate by prodigiosin **1** is consistent with both H^+/Cl^- symport and OH^-/Cl^- antiport mechanisms.

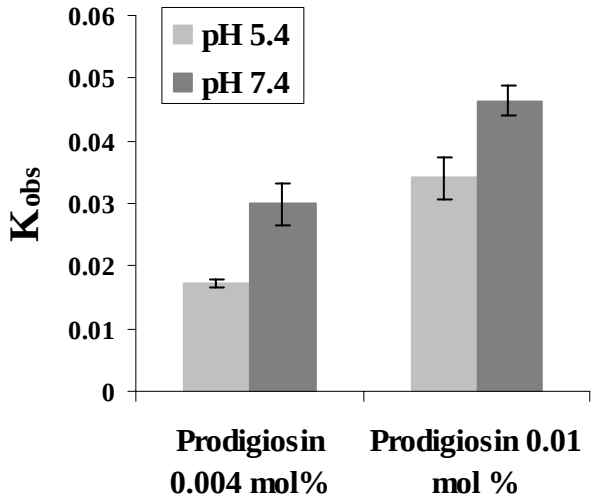

Figure S4. The influence of pH on chloride transport by prodigiosin **1**. Determined using entrapped lucigenin dye in a chloride gradient assay on EYPC liposomes in 10 mM sodium phosphate buffer containing 75 mM Na_2SO_4.

EYPC liposomes for comparative chloride gradient assays:

A. Lucigenin dye, Na_2SO_4. Data obtained with these liposomes is in Fig. 2A of the paper.

A thin film of 50 mg EYPC was prepared by evaporation of a chloroform/methanol solution of the lipid and then further removal of solvent *in vacuo* for 2 h. The film was hydrated with 1 mL of 10 mM sodium phosphate containing 1 mM lucigenin and 75 mM Na_2SO_4 at pH 6.4. After 5 freeze/thaw cycles, the vesicles were extruded 21 times through a 100 nm polycarbonate membrane and then passed through a

Sephadex (G-25) column to remove external dye. The isolated liposomes were diluted to a final concentration of 11 mM.

B. HPTS dye, Na_2SO_4. Data obtained with these liposomes is in Fig. 2B of the paper. Liposomes were prepared using the procedure described above, except the internal solution contained 10 μM HPTS (pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt)[3,7] as opposed to 1 mM lucigenin.

C. Lucigenin dye, $NaNO_3$. Data obtained with these liposomes is in Fig. 2C of the paper. Liposomes were prepared as described above in part A, except the internal solutions contained 100 mM $NaNO_3$ as opposed to 75 mM Na_2SO_4. (Note that 100 mM $NaNO_3$ and 75 mM Na_2SO_4 are used because they give liposome systems that are iso-osmotic.)

D. HPTS dye, $NaNO_3$. Data obtained with these liposomes is in Fig. 2D of the paper. Liposomes were prepared using the procedure described above in part C, except the solution contained 10 μM HPTS as opposed to 1 mM lucigenin.

Representative Assay: In a typical experiment, 0.1 mL of the liposome solution was diluted into 2 mL of a solution of 10 mM sodium phosphate (pH 6.4), 100 mM $NaNO_3$ to which 4 μL of 0.05 mM prodigiosin solution was added, giving a 1:5,000 ligand to lipid ratio (or 0.004 mol %). Fluorescence was monitored for 500 s. At t=20 s, 0.02 mL of 4._ M NaCl was added to the cuvette through an injection port to give a final concentration of 25 mM chloride outside of the liposomes. At t=470 s, 0.04 mL of 10 % Triton-X detergent was to destroy the liposomes and determine maximal fluorescence quenching

of lucigenin. Experiments were repeated in triplicate and all traces reported are the average of the three trials. Chloride concentration (Figure 2A,C of the paper) was determined from the fluorescence of lucigenin using the Stern-Volmer constant obtained as in **Figure S1**. The pH (Figure 2B,D of the paper) was determined from the fluorescence ratio of HPTS in the protonated and deprotonated state.[3] The fluorescence of ruptured HPTS liposomes in buffer solutions at known pH was plotted as a graph of $Log(F_1/F_0)$ vs. pH to derive a standard equation for determining pH from experimentally observed fluorescence ratios in a dual wavelength assay. This plot has been reported previously[7] and the derived equation is $pH=1.1684*Log(F_1/F_o)+6.9807$.

References for Supporting Information.

1. For a detailed description of the extrusion technique see the Avanti website: www.avantilipids.com/PreparationOfLiposomes.html.
2. For a description of Stern-Volmer determination: F. Wissing, J. A. C. Smith, *J. Membr. Biol*. 2000, **177**, 199; other Lucigenin references: J. Biwersi, B. Tulk, A. S. Verkman, *Anal. Biochem.* 1994, **219,** 139; J. Jiang, Y. Song, C. Bai, B. H. Koller, M. A. Matthay, A. S. Verkman, *J. Appl. Physiol*. 2003, **94**, 343.
3. V. Sidorov, F. W. Kotch, G. Abdrakhmanova, R. Mizani, J. C. Fettinger, J. T. Davis, *J. Am. Chem. Soc*. 2002, **124**, 2267.
4. A V. Koulov, T. N. Lambert, R. Shukla, M. Jain, J. M. Bood, B. D. Smith, H. Y. Li, D. N. Sheppard, J. B. Joos, J. P. Clare, A. P. Davis *Angew. Chem., Int. Ed.*, 2003, **42**, 4931; G. Deng, T. Dewa, S. L. Regen, *J. Am. Chem. Soc.* 1996, **118**, 8975.

5. W-H. Chen, X. B. Shao, S. L. Regen *J. Am. Chem. Soc.* 2005, **127**, ASAP 10.1021/ja053527q.

6. pH dependence of prodigiosin and analogs: T. Sato, H. Konno, Y. Tanaka, T. Katoaka, K. Nagai, H.H. Wasserman, S. Ohkuma, *J. Biol. Chem.* 1998, **273**, 21455. J.L. Sessler, L.R. Eller, W.-S. Cho, S. Nicolaou, A. Aguilar, J.T. Lee, V.M. Lynch, D.J Magda, *Angew. Chem. Int. Ed*. 2005, 45, manuscript in press.

7. V. Sidorov, F. W. Kotch, J. L. Kuebler, Y.-F. Lam, J. T Davis, *J. Am. Chem. Soc*. 2003, **125**, 2840. See the experimental section.